The Invention

The invention is directed to recombinant materials which encode single-chain forms of four specific hormones: LH, FSH, TSH and CG. These single-chain forms retain the biological activity of the heterodimers that are the native forms of these glycoproteins, unless they are specifically designed to be antagonists. The single-chain forms may be used in a manner similar to that associated with the native protein. The possible advantages of the single-chain forms are set forth on page 6 of the specification beginning at line 7. In any event, it is an unpredictable result that it is possible to construct single-chain forms of these hormones which retain the biological activity of the heterodimeric counterpart.

The Rejections

The sole remaining rejection is made with respect to the combination of Thomason with two Reddy documents. In some instances, specific claims are rejected on this basis with the addition of still another document(s). These additional documents will be discussed below. However, in all cases applicants believe the rejection is in error because first, there is no motivation to combine Thomason with the two Reddy documents, and, second, even if these documents were combined, making the invention obvious to try, there is no reasonable expectation of success.

With respect to motivation, the Office is reminded that the motivation to combine these documents cannot come from hindsight but must be suggested either by the documents themselves or by the problem to be solved. The basis of this principle in the decided case law was extensively discussed in the previous response and is incorporated herein by reference. The Office appears to take the view that the motivation here lies in the problem to be solved. But this is not the case. While the advantages of a single-chain form as set forth on page 6 of the application refer to problems of recombinant production, and mention bacteria, the major advantages cited are enhanced stability, enhanced half-life, and value as a research tool to explore the effect of varying the molecule. Typically, the heterodimers representing the wild-type proteins corresponding to the single-chain forms of the present invention are produced in mammalian cells, typically CHO cells. There is no problem here with renaturation, which is the specific problem that Thomason sets out to solve.

This is apparent from Thomason, column 2. The discussion there makes clear that Thomason regards expression in yeast and mammalian cells unsatisfactory because of low yields and the requirement for more fastidious culture conditions. The problem to be solved in Thomason is to provide at least a partial solution to the problem of refolding when the PDGF is formed in bacterial cells where yields are higher. This is a particular problem for PDGF for two reasons. First, PDGF can spontaneously form homodimers as well as heterodimers. This is not true with respect to the glycoprotein hormones. Second, and perhaps more relevant, the incorrect conformations of PDGF, resulting in inclusion bodies in bacterial production, are actually held together by disulfide bonds which must be broken in order for refolding to occur. The advantages of the single-chain form, then, according to Thomason, are simply that "[T]he reactions necessary to generate the biologically active multimeric form of the polypeptide proceed with first order rather than second or higher order reaction kinetics. The fusion multimers of the present invention also eliminate the simultaneous formation of undesired polypeptide byproducts during refolding." (Column 2, line 60 *et seq.*) These problems are simply not those addressed by the present invention. There is no problem with formation of homodimers; refolding is not necessary when the glycoproteins are (as disclosed by Reddy) recombinantly produced as they conventionally are in mammalian cells.

Further discussion of the problems to be solved by Thomason is set forth in column 5, beginning at line 26. This section specifically points out that when the subunits are prepared separately in bacteria and refolded, where a PDGF-AB heterodimer is desired, side products containing PDGF-AA and PDGF-BB are also formed. This is no doubt aggravated by the several mature forms of at least the B subunit known to be available (see column 5, lines 9-18). These problems are recognized to be present even if mammalian or yeast host cells were used for expression as Thomason states in column 5, lines 44-50. *None of these problems exists or needs to be solved with respect to the glycoprotein hormones.* They do not form homodimers spontaneously or under refolding conditions and they do not have miscellaneous mature forms of the β-subunit.

Certainly Thomason does not itself suggest that its disclosure should be combined with descriptions of other unrelated proteins. In column 6, lines 31-38, the reader is directed only to other members of the PDGF family.

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Thus, applicants fail to see any motivation to apply the techniques disclosed in Thomason to the glycoprotein hormones disclosed in the secondary Reddy references, absent the disclosure of the invention herein. As the Office is almost certainly aware, the teaching of a document needs to be considered as a whole, not only those portions that support the position of the Office. Taken as a whole, Thomason merely suggests that where problems arise through the necessity to refold multimers made up of subunits where undesired side products arise due to the tendency to form both homodimers and heterodimers, and where the subunits are linked through disulfide bonds (column 3, lines 45-53), it may be advantageous to prepare the compound, specifically a member of the PDGF family, as a single-chain fusion protein.

Applicants are aware that Thomason, which is cited under 35 U.S.C. § 102(e), is accorded full status as a document citable under 35 U.S.C. § 103 according to the holding in *Hazeltine v. Brenner*, 147 USPQ 429 (S.Ct. 1965). However, in evaluating motivation to combine, the Office is asked to consider that as of the priority date of the present application, there could have been *no possible* motivation to combine Thomason with anything else since Thomason was not a public document at that time.

Even if Thomason is, however, combined with Reddy and it would have been "obvious to try" to make single-chain forms of the glycoprotein hormones which retain their biological activity, there was clearly no reasonable expectation of success. As verified by the enclosed Declaration of Dr. Aaron Hsueh, it is impossible to predict from one protein to the next whether formation of a single-chain moiety from a heterodimer would or would not result in a similarly biologically active protein.

As set forth in Dr. Hsueh's Declaration, constructs were made to produce a single-chain form of the gonadal glycoprotein hormone inhibin which regulates pituitary FSH secretion. Enclosed herewith, for the convenience of the Office, is an article by Mason, A.J. *et al. Biochem Biophys Res Commun* (1986) 135:957-964, which describes the precise structure of inhibin. As shown in Figure 1, the mature 134-amino acid α-inhibin subunit is formed from a much longer protein containing 335 amino acids. Similarly, the two alternative β-subunits are obtained from longer precursor proteins. As set forth in Dr. Hsueh's Declaration, a construct was prepared wherein the 3' end of the nucleotide sequence encoding the mature β-subunit was fused to the 5' end of the mature α-subunit which did not contain the pro domain of the α-subunit. No secretion of the single-chain protein putatively expressed was even obtained. It will be noted, of course,

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that Thomason specifically teaches that in the case of a PDGF fusion dimer, a spacer moiety is not believed to be necessary. (Column 6, lines 39-40 and 59-60.) Dr. Hsueh then constructed a nucleotide sequence and expression system for a fusion protein where the α-subunit pro sequence was included as a "spacer" between the β-subunit and the downstream α mature subunit (said to be unnecessary by Thomason). Protein was secreted, but was not biologically active. These results are in direct contradiction to the results obtained by Thomason who was, apparently, able to show mitogenic activity by the fusion dimer prepared using two B subunits separated by a spacer.

The contrast with the results of Thomason is even more striking by virtue of the fact that inhibin, like PDGF, is composed of subunits covalently linked by disulfide bonds. Thus, one would expect the predictability of success for inhibin to be even greater than that for the glycoprotein hormones which contain disulfide links only to constrain the three-dimensional structure within the individual subunits. This adds to the uncertainty since fusion in a single-chain form could conceivably interfere with this intrasubunit binding, whereas it would be less likely to interfere with the intersubunit binding that is responsible for stabilizing the dimer.

Thus, Dr. Hsueh's Declaration clearly shows that even if single-chain glycoprotein hormones which retain biological activity (or could be designed to become antagonists) were obvious to try on the basis of Thomason, there is no reasonable expectation of success. A more closely related protein to PDGF such as inhibin fails to behave as putatively predicted by Thomason.

The results obtained by Dr. Hsueh are clearly in agreement with the views expressed by experts in the field of protein chemistry and manipulation. The enclosed Declaration of Dr. Elliot L. Elson, a prominent expert in the field, verifies that results obtained with one protein or one type of protein do not extrapolate to proteins of other kinds. Specifically, extrapolating successful results with multimeric proteins wherein the subunits are stabilized by disulfide bonds does not predict success with multimers where subunits are noncovalently associated. The reason for this is that, because of the stability of the disulfide linkages relative to noncovalent association, the distortions in activity caused by forcing the subunits into association are inherently greater for noncovalently bonded subunits than for covalently bonded ones. Hence, it is entirely unpredictable, based on the disclosure of Thomason, whether multimers other than those in the PDGF family, even when disulfide-bonded, would retain activity and suitability for

administration; the unpredictability skyrockets when an attempt is made to extrapolate Thomason's conclusions to multimers where the subunits are noncovalently associated.

Also enclosed, as a matter of interest, is an article by Grossmann, M. *et al. J Biol Chem* (1997) 272:21312-21316 which credits one of the present applicants as having "pioneered" the construction of the bioactive gonadotropins as single chains. These authors, in addition, seem unwilling even to extrapolate the results with the gonadotropin to the closely related TSH. However, the article does show that, as predicted by the present applicants, the related protein TSH can be prepared in single-chain form successfully.

For the foregoing reasons, all of the pending claims are patentable over the above-cited art.

The Office apparently acknowledges this, at least, with respect to certain claims. With respect to claims 7, 9, 17, 19, 27, 29, 37 and 39, which provide that variants have particular types of alterations, the Office combines the primary and secondary references with Zurawski *et al.* The Office asserts that techniques taught by Zurawski of structural deletion analysis to identify critical regions within mouse IL-2 could equally be applied to the glycoprotein hormones. The motivation to combine Zurawski is said to reside in Zurawski's teaching of the general utility of the method. This hardly amounts to motivation. It does not identify a problem to be solved or provide a reason to modify the glycoprotein hormones. Even if Zurawski is combined with the remaining cited references, there is no showing on the part of the Office that the specific deletions or substitutions required would result.

Claims 3, 6, 13, 36, 23, 26, 33 and 36 were rejected on the same basis with the addition of Fares *et al.* or Boime, U.S. Patent No. 5,585,345. These claims require the inclusion of a partial or complete CTP in the single-chain form. Again, there appears to be no motivation to combine the teaching of these tertiary references with those already cited absent the teaching of the present invention.

Claims 5, 15, 25 and 35 were rejected adding in the citations to Chaudhary *et al.* and Cousens *et al.* These claims require a gly/ser repeat. The relevance of Chaudhary is unclear; the mere inclusion of a 15-residue long stretch of gly and ser residues in a much longer linker hardly suggests any focus on that particular sequence. As for Cousens, column 4 fails to suggest gly/ser linkages at all. Gly and ser and included in a very long list of appropriate amino acids (column 4, lines 34-35). The exemplified linkers in Cousens are not gly/ser linkages either (lines 36-38).

Conclusion

There is no motivation to combine Thomason which is directed to a method to assist refolding of disulfide-linked dimeric proteins produced recombinantly in bacteria with the disclosure of Reddy which merely describes recombinant materials for the production of FSH in murine cells. The glycoprotein hormones further do not exhibit the problems said to be resolved by Thomason in the case of PDGF -- i.e., inappropriate formation of unwanted homodimers or dimers with the "wrong" mature subunit. Even if the documents were combined, there is no reasonable expectation of success. The unpredictability of success has been confirmed by the sworn testimony of two experts in the field. Further, there is no motivation to combine any of the tertiary references with the Thomason/Reddy combination. Accordingly, it is believed that all pending claims are in a position for allowance and passage of these claims to issue is respectfully requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 295002005025. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

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Human Thyroid-stimulating Hormone (hTSH) Subunit Gene Fusion Produces hTSH with Increased Stability and Serum Half-life and Compensates for Mutagenesis-induced Defects in Subunit Association*

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The human thyroid-stimulating hormone (hTSH) subunits α and β are transcribed from different genes and associate noncovalently to form the bioactive hTSH heterodimer. Dimerization is rate-limiting for hTSH secretion, and dissociation leads to hormone inactivation. Previous studies on human chorionic gonadotropin (hCG) and human follicle-stimulating hormone had shown that it was possible by subunit gene fusion to produce a bioactive, single chain hormone. However, neither the stability nor the clearance from the circulation of such fused glycoprotein hormones has been studied. We show here that genetic fusion of the hTSH α- and β-subunits using the carboxyl-terminal peptide of the hCG β-subunit as a linker created unimolecular hTSH whose receptor binding and bioactivity were comparable to native hTSH. Interestingly, the fused hTSH had higher thermostability and a longer plasma half-life than either native or dimeric hTSH containing the hCG β-subunit-carboxyl-terminal peptide, suggesting that dimer dissociation may contribute to glycoprotein hormone inactivation *in vivo*. In addition, we show for the first time that synthesis of hTSH as a single polypeptide chain could overcome certain mutagenesis-induced defects in hTSH secretion, therefore enabling functional studies of such mutants. Thus, in addition to prolongation of plasma half-life, genetic fusion of hTSH subunits should be particularly relevant for the engineering of novel analogs where desirable features are offset by decreased dimer formation or stability. Such methods provide a general approach to expand the spectrum of novel recombinant glycoprotein hormones available for *in vitro* and *in vivo* study.

Thyroid-stimulating hormone (TSH)[1] belongs to the glycoprotein hormone family, which also includes the gonadotropins follicle-stimulating hormone (FSH), luteinizing hormone, and chorionic gonadotropin (CG). These hormones exist as heterodimers composed of a common α-subunit, which is noncovalently linked to a hormone-specific β-subunit (1). Crystallization of hCG had revealed that both subunits have a similar overall structure with a central cystine knot motif (2, 3). Therefore, the glycoprotein hormones are now considered members of the cystine knot growth factor superfamily that includes a variety of structurally related dimeric growth factors, such as nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, and transforming growth factor-β (4, 5). The glycoprotein hormone α-subunit is encoded in a single gene and thus identical in the amino acid sequence within a species. In contrast, the β subunits are unique, encoded in distinct genes and responsible for biological specificity (6, 7).

Assembly of the α- and β-subunits is an early posttranslational event in glycoprotein hormone synthesis occurring in the endoplasmic reticulum (8). Heterodimerization is critical for disulfide bond formation and for hormone-specific posttranslational modifications, such as processing of the carbohydrate side chains, and thus rate-limiting for the secretion of glycoprotein hormones (9, 10). Moreover, dimer formation is essential for hormonal activity, since free subunits have minimal receptor binding affinity (1). In addition, dissociation of heterodimeric glycoproteins into their subunits may be a significant factor in terminating glycoprotein hormone activity *in vivo* (11).

Therefore, covalent linking of the glycoprotein hormone subunits should overcome assembly-dependent deficiency in secretion and may increase hormone stability and activity. It has recently been pioneered by Boime and colleagues and subsequently shown by the group of Puett that bioactive gonadotropins could be produced as single chains (12–14), but it is not clear whether this approach is applicable for hTSH, or whether such fusion would affect the stability or the *in vivo* clearance of these hormones. Such fusion should be particularly relevant to TSH, since the free TSH β-subunit, in contrast to the free CG β-subunit, is unstable in the monomeric form and degraded intracellularly unless stabilized by dimerization with the α-subunit (15). Here, we show that it is possible by subunit gene fusion to produce a tethered form of hTSH with comparable *in vitro* activity to dimeric hTSH. Furthermore, fusion significantly increased the stability and prolonged the *in vivo* half-life of hTSH. Moreover, the expression of hTSH as a single chain could overcome selected mutagenesis-induced defects in hTSH secretion, and thus, this approach may be used to expand the spectrum of structure-function studies of glycoprotein hormone analogs. Subunit gene fusion therefore appears to be a promising strategy, not only for the generation of long lasting

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[1] The abbreviations used are: TSH, thyroid-stimulating hormone; hTSH, human thyroid-stimulating hormone; hTSHβ, human thyroid-stimulating hormone β subunit; CG, choriogonadotropin; FSH, follicle-stimulating hormone; rh, recombinant human; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; wt, wild type; CTP, carboxyl-terminal peptide; SC, single chain; CMV, cytomegalovirus.

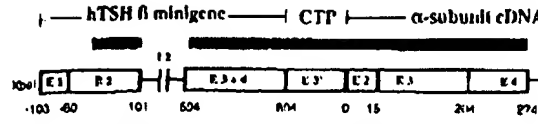

FIG. 1. **hTSH-SC construct.** The hTSHβ minigene bearing the 32-amino acid CTP of the hCGβ-subunit was fused to the α-subunit cDNA by overlap extension PCR as described under "Experimental Procedures." *E*, exon; *I*, intron. The numbers below denote the base pairs corresponding to the respective subunit genes and the *gray bar* above represents the coding region of the mature protein.

hTSH analogs, but particularly for the development of recombinant mutants with desirable characteristics, the utility of which may be limited by decreased stability.

EXPERIMENTAL PROCEDURES

Materials—CHO cells stably transfected with the hTSH receptor (clone JP09) were kindly donated by Dr. G. Vassart, Belgium, and FRTL-5 cells expressing the endogenous rat TSH receptor by Dr. L. D. Kohn, Interthyr Research Foundation (Baltimore, MD). cAMP antibody was generously supplied by Dr. J. L. Vaitukaitis, National Institutes of Health (Bethesda, MD). Cell culture media and reagents were purchased from Life Technologies, Inc., and ^{125}I-cAMP and ^{125}I-hTSH radiolabeled to a specific activity of 40–60 μCi/μg from Hazleton (Vienna, VA). PCR reagents were obtained from Boehringer Mannheim and New England Biolabs (Beverly, MA).

Site-directed Mutagenesis—The construction of the hTSH β subunit bearing the carboxyl-terminal extension peptide of the hCG β-subunit (hTSHβ-CTP) has been described previously (16). To produce single chain hTSH (hTSH-SC), we used overlap extension PCR (17) to fuse the amino terminus of the α-subunit cDNA (without the signal sequence) to the carboxyl-terminal end of the hTSHβ-CTP (Fig. 1). Primers P2 5′-CAC ATC AGG AGC TTG TGG GAG GAT CGG and P3 5′-ATC CTC CCA CAA GCT CCT GAT CTG CAG span both the carboxyl-terminal end of the hTSH β-subunit containing the hCGβ-CTP (hTSHβ-CTP) as well as the amino terminus of the coding sequence of the α-subunit. In addition, P1 5′-CTC GAG TCT AGA ATG ACT GGT CTC TTT CTG ATG was designed to anneal 5′ of the hTSH CTP minigene signal peptide, and P4 5′-CGA CGT GGA TCC ATG CTG TAT TCA TTC to anneal 3′ of the hTSH β-subunit cDNA. Initially, two PCR reactions were performed: P1 and P2 were used with the hTSH-CTP as the template (PCR no. 1), and P3 and P4 using the α-subunit cDNA (PCR no. 2). In a third PCR reaction (PCR no. 3), both these overlapping products were used as combined template to generate the single chain hTSH-SC with P1 and P4.

To create Gln^{56}-Gln^{78}/TSHβ-SC in which both α glycosylation recognition sequences were deleted by mutating both Asn^{56} and Asn^{78} to Gln, a previously described α-subunit cDNA construct (Gln^{56}-Gln^{78}) (18) was used as the template for PCR no. 2. Similarly, to obtain Asp^{30}/TSHβ-SC, the α-subunit cDNA construct Asp^{30} (19) served as the template in PCR no. 2. Following subcloning of the fused wild type or mutant 2-kilobase pair hTSH-SC constructs into the pLB-CMV expression vector, the entire PCR product was sequenced in each case to rule out any undesired polymerase errors.

Transient Expression—CHO-K1 Cells (ATCC, Rockville, MD) were maintained in Ham's F-12 medium supplemented with 5% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and glutamine (4 mM). To obtain dimeric wild-type hTSH (hTSH-wt), cells were cotransfected in 60-mm culture dishes with the α-subunit cDNA in pcDNA I/neo and the hTSHβ minigene in the pLB-CMV vector, using a total amount of 2 μg DNA per dish and a liposome formulation (LipofectAMINE reagent, Life Technologies, Inc.) as described previously (20). The hTSH-SC fusion products in the pLB-CMV vector were transfected with identical amounts of total DNA. On the following day, the transfected cells were transferred to CHO serum-free medium (Life Technologies, Inc.). After an additional 48 h, the supernatants, including control medium from mock transfections using the expression plasmids without gene inserts, were harvested. The collected media were then concentrated using a Centriprep 10 concentrator (Amicon, Beverly, MA) and used for immunoassays, the various activity assays, and clearance studies.

Immunoassays of hTSH—The hTSH constructs were quantified with a panel of different immunoassays, using a total of four different hTSH immunoassays utilizing different monoclonal antibodies, which were described in detail previously (19, 21).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Conditioned media from transiently transfected CHO cells were concentrated, fractionated on ConA-Sepharose columns (Pharmacia), reconcentrated, and denatured by boiling in 0.25% SDS, 0.5% β-mercaptoethanol. Samples were then resolved on 14% Tris-glycine polyacrylamide gels, transferred to nitrocellulose membranes, and incubated overnight with a polyclonal rabbit antibody directed against the hTSH α-subunit (18). Antigen-antibody complexes were subsequently visualized by chemiluminence using a horseradish peroxidase-coupled anti-rabbit IgG and a luminol substrate (Boehringer Mannheim).

Radioreceptor Assay of hTSH—The receptor-binding activity of the various hTSH constructs was determined by their ability to displace ^{125}I-bTSH from solubilized porcine thyroid membrane receptor preparations (Kronus, Dana Point, CA) following the manufacturer's instructions. Binding was also studied in whole cells using FRTL-5 cells expressing the endogenous rat TSH receptor, as described previously (20).

cAMP Production in JP09 Cells—CHO cells stably expressing the rhTSH receptor (JP09) were grown in 96-well culture plates in Ham's F-12 medium supplemented as above. Confluent cells were incubated for 2 h at 37 °C, 5% CO_2, with serial dilutions of hTSH constructs or control medium from mock transfections in a modified Krebs-Ringer buffer supplemented with 280 mM sucrose to maintain isotonicity and 1 mM 3-isobutyl-1-methylxanthine. The amount of cAMP released into the medium was determined by radioimmunoassay (20).

cAMP Production in FRTL-5 Cells—FRTL-5 cells were maintained as described elsewhere (20) and, prior to the cAMP assay, grown in 96-well culture plates in the absence of TSH for 6–8 days. cAMP production of hTSH constructs was determined using the protocol for JP09 cells.

Growth Assay in FRTL-5 Cells—FRTL-5 cells were grown in 24-well plates in the presence of TSH to 30% confluence and then cultured in TSH-free medium for 4 days. Subsequently, the cells were incubated with serial dilutions of hTSH constructs or control from mock transfected cells. After 48 h, 1.0 μCi of [^{3}H]thymidine per well (DuPont) was added, and the cells were incubated for an additional 24 h. Subsequently, [^{3}H]thymidine uptake measured as described previously (18).

Plasma Clearance Rate—The clearance rate of the hTSH constructs was determined in the rat after intravenous injection of the different hTSH preparations and subsequent determination of hTSH serum levels at defined intervals from 1 to 120 min. Experimental details of this procedure have been described previously (22, 23).

RESULTS

Genetic Fusion of the hTSH α- and β Subunit—Truncation as well as amino acid mutation studies had previously indicated the importance of the α-carboxyl terminus for hTSH activity (20). To maintain accessibility of this region, we fused the carboxyl terminus of the TSH β-subunit to the amino terminus of the α-subunit. We also included the CTP of the hCG β-subunit, here defined as the carboxyl-terminal 32 amino acids of the hCG β-subunit. The CTP has a high proline/serine content, which lacks significant secondary structure and was previously shown to be suitable as a flexible linker for efficient expression of single chain hFSH (13). In keeping with previous observations, addition of CTP to the hTSH β-subunit was predicted not to affect receptor binding or intrinsic activity of hTSH (16). Since addition of the CTP had previously been shown to prolong the half-life of hTSH, the clearance rate of hTSH-SC was compared with both dimeric hTSH-wt as well as hTSH-CTP (see below).

Effect of Subunit Fusion on hTSH Secretion—To demonstrate that hTSH-SC was indeed produced and secreted as a single chain, we performed SDS-polyacrylamide gel electrophoresis and subsequent Western blotting of ConA-fractionated conditioned media from CHO cells transiently transfected with either the fusion product or individual hTSH subunits using an antibody against the α-subunit. Under reducing conditions, heterodimeric hTSH-wt dissociated into individual subunits, and the free α-subunit migrated at the expected 25 kDa. In contrast, the α-subunit antibody recognized a 55-kDa band consistent with the covalently linked hTSH fusion protein (Fig. 2). The level of secretion of hTSH-SC from transiently transfected CHO cells, as determined by four different immunoassays, was similar to hTSH-wt (Table I), if individual subunit plasmids were cotransfected at a 3 to 1 molar ratio. Such

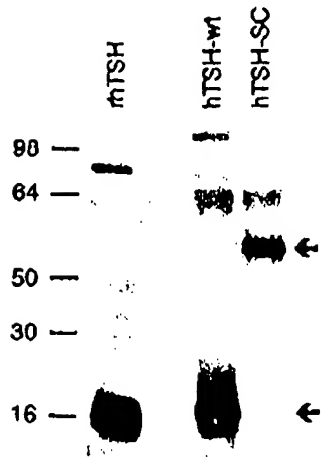

FIG. 2. **Western blot analysis of ConA-Sepharose-fractionated hTSH-wt and hTSH-SC obtained from conditioned media harvested from transiently transfected CHO cells. Also shown, as an internal standard, is rhTSH, kindly provided by the Genzyme Corp. (Cambridge, MA). A polyclonal rabbit antibody against the hTSH α-subunit was used. Under the reducing conditions used, dimeric hTSH dissociates into individual subunits, and the free α-subunit migrated as the expected 25-kDa protein (*bottom arrow*). In contrast, the hTSH-SC migrated at 55 kDa, consistent with the size of a linked α-β-subunit complex (*top arrow*). The presence of nonprominent higher molecular weight bands was consistently observed with the different hTSH preparations as well as mock transfected supernatant from independent transient transfections and therefore most likely due to nonspecific antibody interaction. Therefore, a potential specific effect on the biological or physical properties of a hTSH preparation would not be expected.**

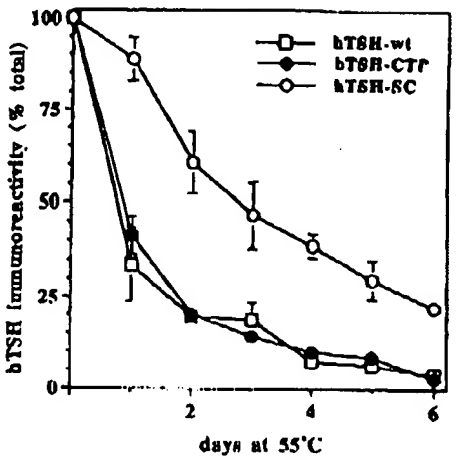

FIG. 3. **hTSH stability.** hTSH immunoreactivity, measured as percent of total remaining hTSH, was determined for hTSH-wt, hTSH-CTP, and hTSH-SC at 55 °C for 6 days using an assay specific for dimeric hTSH without cross-reactivity to free subunits. Values are the mean ± S.E. of three independent experiments, each performed in duplicate. At 37 °C, all hTSH constructs were stable (<5% degradation) for at least 21 days. In some cases, no *error bar* is visible because it is equivalent to or smaller than the size of the respective symbol.

TABLE I
Secretion of hTSH analogs

Hormone levels were determined in conditioned media from CHO cells transiently transfected with the respective hTSH constructs. *n* denotes number of independent transfections, each performed in triplicate dishes. Free α-subunit levels were similar in conditioned media from hTSH-wt and $Asp^{\alpha 38}$/hTSHβ-wt, but not detectable in media from either $Gln^{\alpha 52}$–Gln^{78}/hTSHβ-wt or $Gln^{\alpha 52}$–Gln^{78}/hTSHβ-SC (see text).

Analog	Subunit plasmid ratio	Secretion	n
		ng/ml ± S.E.	
hTSH-wt	α:β 3:1	31.2 ± 1.7	5
	α:β 1:1	24.1 ± 1.6	3
hTSH-CTP	α:β 3:1	25.2 ± 2.8	5
	α:β 1:1	17.1 ± 1.1	3
hTSH-SC		29.6 ± 4.7	11
$Gln^{\alpha 52}$–Gln^{78}/hTSHβ-wt		0.05 ± 0.05	4
$Gln^{\alpha 52}$–Gln^{78}/hTSHβ-SC		5.1 ± 1.8	5
$Asp^{\alpha 38}$/hTSHβ-wt		<0.01	3
$Asp^{\alpha 38}$/hTSHβ-SC		<0.01	3

a 3 to 1 molar excess of the α-subunit plasmid led to a higher secretion of dimeric hTSH compared with transfection of both subunits at an equimolar ratio. Addition of the CTP to the hTSH β-subunit reduced secretion of dimeric hTSH, whereas fusion of the hTSH subunits with inclusion of the CTP sequence as a linker did not impair subunit folding or expression of the hormone (Table I).

Effect of Subunit Fusion on Secretion-deficient hTSH α-Subunit Mutants—To test the effects of subunit fusion on mutagenesis-induced defects in hTSH secretion, we studied the secretion of single chain hTSH analogs $Gln^{\alpha 52}$–Gln^{78}/TSHβ-SC lacking the two α-subunit glycosylation recognition sequences and $Asp^{\alpha 38}$/hTSHβ-SC. These mutations had previously been shown to profoundly decrease or abolish the secretion of dimeric hTSH (18, 19) (Table I). Consistent with findings that non-glycosylated glycoprotein hormone subunits are misfolded and degraded intracellularly, the free $Gln^{\alpha 52}$–Gln^{78} subunit was not detectable (<0.01% of hTSH-wt-free α-subunit) by an α-subunit-specific radioimmunoassay. In contrast, free $Asp^{\alpha 38}$ subunit was secreted in levels quantitatively similar to hTSH-wt free α-subunit (19), suggesting that the failure of the $Asp^{\alpha 38}$ subunit to dimerize with hTSH β-subunit was not related to its misfolding or degradation. Fusion of the $Gln^{\alpha 52}$–Gln^{78} subunit to the hTSH β-subunit increased secretion, indicating that fusion of both subunits can partially overcome the requirement of α-subunit carbohydrate chains for hTSH secretion. In contrast, fusion of the $Asp^{\alpha 38}$ subunit did not increase the amount of hTSH produced, suggesting that this particular mutation, possibly due to its predicted location at the subunit interface in close proximity to residues forming intersubunit hydrogen bonds (2, 3) prevents subunit association (Table I).

Effect of Subunit Fusion on hTSH Stability—Stability of the different hTSH proteins was tested initially by incubating conditioned media obtained from transient transfections at 37 °C. All three forms of hTSH, hTSH-wt, hTSH-CTP as well as hTSH-SC were stable at this temperature, and there was minimal (<5%) degradation over a period of 21 days, as judged by repeated determinations of hTSH immunoreactivity with an assay specific for heterodimeric hTSH, which does not recognize free subunits. However, incubation at 55 °C showed that the fused hTSH-SC was significantly more stable than dimeric hTSH in that less than 16% of hTSH-SC was degraded after 24 h, compared with more than 50% of dimeric hTSH, either hTSH-wt or hTSH-CTP (Fig. 3).

Effect of Subunit Fusion on Receptor Binding and Intrinsic Activity of hTSH—The receptor binding of the fused hTSH-SC was similar to that of hTSH-wt and hTSH-CTP when tested in porcine thyroid membranes (Fig. 4) or in FRTL-5 cells expressing the endogenous rat TSH receptor (not shown). In addition, the ability of hTSH-SC to induce cAMP stimulation in JP09 cells (Fig. 5*a*), as well as cAMP stimulation (Fig. 5*b*) and growth promotion (Fig. 5*c*) in FRTL-5 cells was comparable to that of hTSH-wt and to that of hTSH-CTP. This indicates that both introduction of the CTP linker as well as subunit fusion did not alter the *in vitro* characteristics of hTSH.

Effect of Subunit Fusion on hTSH Clearance—In accord with previous studies from our laboratory (16), addition of the CTP to the hTSH β-subunit significantly prolonged the plasma half-life of dimeric hTSH. 50% of the hTSH-CTP was cleared from

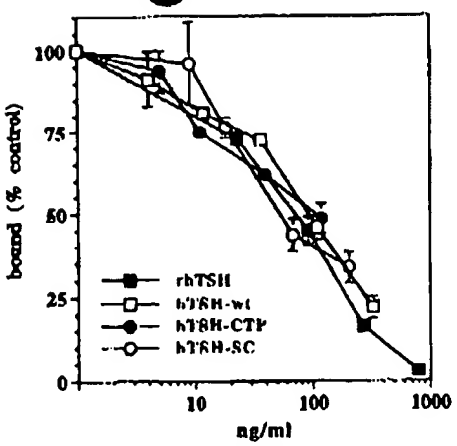

FIG. 4. **Inhibition of ^{125}I-bTSH receptor binding by the hTSH preparations.** Increasing doses of hTSH were incubated with porcine membranes in the presence of a constant amount of ^{125}I-bTSH. ^{125}I-bTSH bound to membranes was precipitated and quantitated in a γ counter. Radioactivity precipitated in the presence of concentrated medium from mock transfections was defined as 100%. rhTSH was obtained from the Genzyme Corp. Values are the mean ± S.E. of three independent experiments, each performed in at least duplicate. Also see legend to Fig. 3.

the rat circulation after 23.2 ± 7.9 min compared with 8.7 ± 6.1 min for hTSH-wt ($p = 0.01$). Remarkably, fusion of the individual subunits including the CTP as a linker led to an even further significant prolongation of half-life; 50% of hTSH-SC was cleared after 61.6 ± 14.4 min ($p = 0.02$ compared with hTSH-CTP) (Fig. 6).

DISCUSSION

The half-life of recombinant analogs can be prolonged by increasing the Stoke's radius of a protein using polyethylene glycolylation or the introduction of new carbohydrate recognition sites, by modification of protease recognition sites to increase stability, or by carbohydrate modification to avoid carbohydrate-specific clearance mechanisms (5, 21, 23). Our present study using a genetically fused, single chain hTSH highlights a novel way by which an increased *in vivo* half-life may be achieved.

Although it had previously been shown that bioactive hCG and hFSH could be produced as a single chain (12–14), the effect of genetic fusion on glycoprotein hormone stability and plasma clearance rate had not previously been investigated. Further, from the findings on hCG and hFSH, it was not predictable whether a fusion approach would also be feasible for hTSH. In particular, recent mutational analysis of hTSH structure-function relationships has identified common α-subunit domains that play strikingly different roles for heterodimer formation, receptor binding, and bioactivity of hTSH compared with hCG and hFSH (18–20). Interestingly, these domains are located in close proximity to the β-seat-belt region, which is crucial for hTSH specificity, suggesting that the seat-belt may direct these common domains to function in a hormone-specific fashion (5, 24).

In light of previous observations (16), validated here, that addition of the CTP with its *O*-linked carbohydrate side chains prolonged hTSH half-life *in vivo*, the full-length CTP was used as a linker for fusing the hTSH subunits. We anticipated that the linker may synergize with the fusion to prolong the half-life of hTSH *in vivo*. Indeed, gene fusion significantly decreased the clearance rate of dimeric hTSH even when compared with dimeric hTSH bearing the CTP. This indicates that dissociation of hTSH into its subunits occurs *in vivo* and contributes to its deactivation, as individual subunits are devoid of *in vivo* activity and rapidly cleared from the circulation (11).

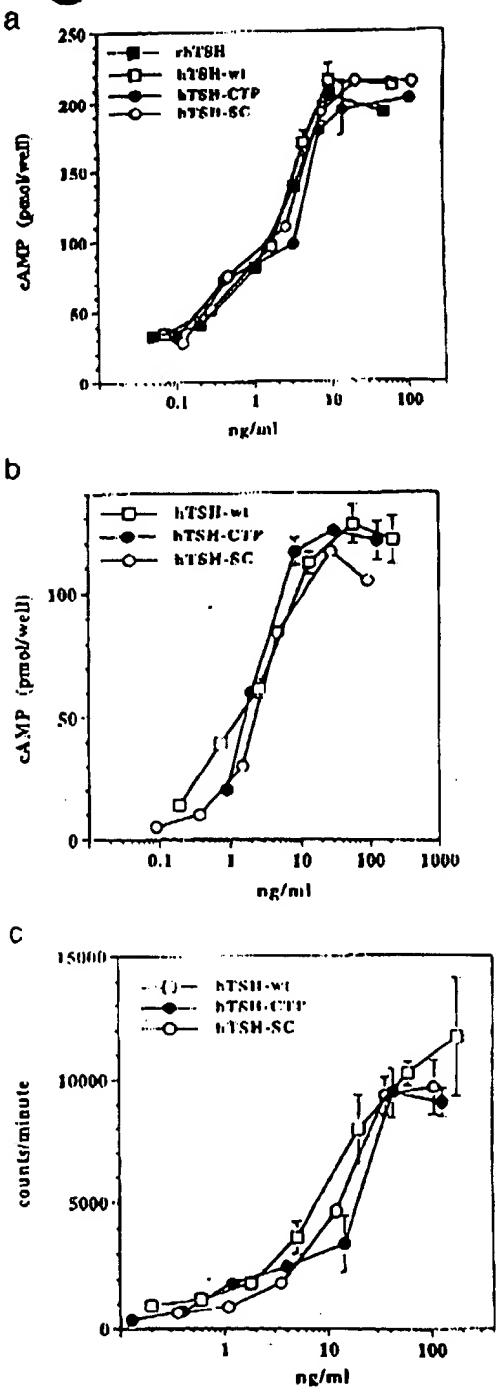

FIG. 5. *a* and *b*, cAMP induction by the various hTSH constructs in JP09 cells (*a*) and FRTL-5 cells (*b*). Increasing concentrations of the various hTSH constructs were incubated with JP09 or FRTL-5 cells, and the cAMP concentration in the resulting supernatants was assayed by radioimmunoassay. *c*, induction of cell growth by the hTSH constructs. Increasing concentrations of hTSH were incubated with FRTL-5 cells, which were previously grown in the absence of TSH. After 48 h, [^{3}H]thymidine was added, and after an additional 24 h, radioactivity incorporated into the DNA was measured. The radioactivity incorporated by the cells in the presence of concentrated medium from mock transfected cells was not different from base line levels. rhTSH represents recombinant hTSH from Genzyme Corp. Values are shown as the mean of triplicate observations ± S.E. See also the legend to Fig. 3.

In addition, fusion of the subunits of hTSH increased its thermostability. It is conceivable that such enhanced stability

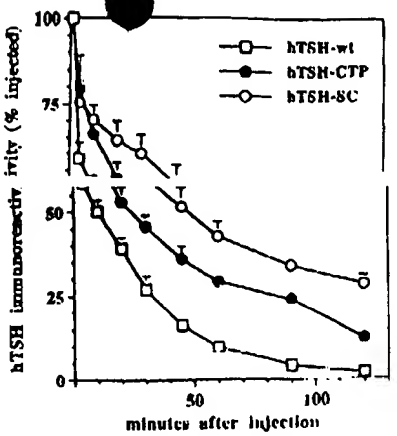

FIG. 6. **Serum disappearance rate of the various hTSH constructs in male rats.** After bolus injection of 200–300 ng of hTSH into the femoral vein, blood for hTSH determinations was obtained over 120 min at equal time points. An immunoradiometric assay without cross-reactivity to rat TSH (Nichols Institute), was used. Immunoreactivity was expressed as mean ± S.E. percent remaining, and serum concentration at 0 min was defined as 100%. A total of $n = 5$ animals was used for each hTSH preparations. See also the legend to Fig. 8.

may become particularly relevant for recombinant glycoprotein hormone analogs with genetically engineered novel features that are less stable than the wild-type hormone. In this respect, hFSH analogs have recently been described, in which site-directed mutagenesis within regions important for activity significantly decreased their stability (25).

Moreover, genetic subunit fusion can overcome certain mutagenesis-induced defects in heterodimer formation. The presence of carbohydrate side chains on both subunits is essential for proper subunit folding and combination, and intracellular assembly of deglycosylated subunits is inefficient (10). Indeed, glycosylation of the α-subunit appears necessary to overcome retention of the hTSH β-subunit in the endoplasmic reticulum (8), and in contrast to the free hCG β-subunit, the free hTSH β-subunit is not efficiently secreted (26). Our fusion experiments suggest that the glycosylated hTSH β-subunit, if fused to an α-subunit devoid of glycosylation recognition sequences, may function as a chaperone inducing α-subunit folding despite the absence of carbohydrate chains and thus partially rescue the nonglycosylated α-subunit. On the other hand, fusion was not able to induce heterodimer formation with a mutated α-subunit Asp^{56} which, although dimer formation-incompetent, nevertheless appeared to be properly folded and secreted.

It is interesting to consider the dimeric structure of glycoprotein hormones from an evolutionary perspective. The glycoprotein hormones were probably derived from a common ancestor gene, and in less developed organisms, a single primordial monomeric hormone with a corresponding receptor was likely sufficient for the necessary endocrine functions (27). To fulfill the requirements for an increasingly complex organism, adopting a dimeric ligand structure enabled functional diversification and increased flexibility without the need for the development of entirely new mechanisms of receptor activation, albeit perhaps at the expense of reduced protein stability. This diversification appears to have evolved by the emergence of inhibitory domains on both ligand and receptor which impose steric hindrances thus allowing only the intended ligand to interact with the common activation domain (28). Such negative specificity determinants have not only developed in glycoprotein hormones and their receptors, but also in other members of the cystine knot growth factor superfamily, such as neurotropins (29), and also in other G protein-coupled receptors (30). More generally, dimer formation is necessary for the activity and specificity of many, if not all cystine knot growth factors, as well as for other bioactive molecules, such as enzymes and transcription factors. In this respect, fusion of individual protein monomers has recently been used to develop transcription factors and cytokine analogs with defined properties and increased biological activities (31, 32). This approach poses a universal strategy to enhance both stability and bioactivity as well as to control specificity of noncovalently linked oligomers, and may also be used to engineer molecules with novel activities or specificities.

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